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14. ABSTRACT
Little of the etiology of childhood leukemia is known; evidence indicates that childhood leukemia is a genetic disease originating *in utero.* Because of the probable multi-factor etiology of leukemia, multi-exposure investigations will contribute more useful information than conducting only single exposure studies. The combination of two environmental exposures, jet propellant-8 (JP-8) and tungsten (W), is unique to military personnel and their families. In the current study, we have used an animal model to investigate the effects of *in utero* exposure to environmental and occupational toxicants (tungsten and JP-8) in combination with infection by MHV-68 virus (murine Epstein-Barr virus) on the development of leukemia-like alterations. Unlike previous results using respiratory syncytial virus, our preliminary results show that exposures using MHV-68 have not resulted in any alterations in blood cell profiles or in spleen sizes. This indicates that the overall response therefore, may be specific for particular viral interactions and is not just a general effect of the JP-8 or tungsten on inflammatory responses. Research is continuing to verify the current preliminary results.

15. SUBJECT TERMS
JP-8 jet fuel, tungsten, Epstein-Barr virus, in utero, lymphocytes, proliferation

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INTRODUCTION

Little of the etiology of childhood leukemia is known; evidence indicates that childhood leukemia is a genetic disease originating *in utero* (e.g. [1-3]). Leukemogenic risk factors are hypothesized to include environmental exposure, genetic predisposition (primarily the prenatal formation of chimeral genes resulting from chromosomal translocations), and viral infection with a probable multi-factorial etiology (reviewed in [4.5]). Environmental exposures that could affect the development of leukemia include metals (especially tungsten) and inhaled jet fuels (JP-8). A summary of our environmental profiling of Fallon, Nevada indicates that the unique portion of the atmospheric, metallic particulate matter is significantly elevated concentrations of cobalt and tungsten which co-vary as a function of time [6,7]. Because of the probable multi-factor etiology of leukemia, multi-exposure investigations will contribute more useful information than conducting only single exposure studies. The combination of two environmental exposures, jet propellant-8 (JP-8) and tungsten (W), is unique to military personnel and their families. Gene microarray data generated from *in utero* tungsten exposure suggests that tungsten may alter immune response to viral influences in mouse pups.

Two hypothetical mechanisms associated with a viral etiology have been postulated for leukemogenesis. Greeves' Hypothesis suggests a post natal exposure to a virus may promote the second genetic hit necessary to cause the leukemia. This could be due to increased inflammation, but more likely due to a delay in immune maturation. This would increase the likelihood that pre-leukemic clones, cells carrying the initial genetic hit as a result of pronounced proliferation during fetal growth, will survive longer and therefore, increase the probability that one of these pre-leukemic clones will evade detection and obtain a second hit during virus-associated inflammation. Alternatively, Smith's Hypothesis suggests that leukemia is associated with a viral exposure occurring *in utero.* We are investigating several hypotheses utilizing exposures of W and/or JP-8 with viruses that have been suggested to be involved in the development of leukemia (cytomegalovirus, Respiratory Syncytial Virus, and specific to this study, a murine model of the Epstein-Barr Virus (MHV-68)).

BODY

As a result of our initial W and virus investigation (in review and Appendix A) conducted in C57BL/6 mice kept immunologically naïve (Greeves' Hypothesis), we have reported that a combination of *in utero* exposure to W and post natal exposure to an infectious agent (Respiratory Syncytial Virus) carrying the peptide signature associated with the HLA supertype associated with pre-B cell leukemia resulted in immune suppression (progressive anemia), neutrophilia (not significant due to small n-values), and splenomegaly ($p=0.0406$, 0.0184, 0.0108 for C, W and RSV, respectively). The study was executed in a general population where the presence or absence of the initial genetic hit and cell-type carrying this hit was not controlled.

In the current study, we are investigating the leukemogenic potential of MHV-68 utilizing both Greeve's and Smith's hypotheses.

- Immune suppression associated with JP8 exposure lends itself most simply to Smith's hypothesis. Therefore, we are investigating whether the immune suppression associated with exposure to JP8 enhances reactivation of MHV-68 and results in leukocytosis and/or splenomegaly in C57BL/6 offspring.
- While the literature does not report that MHV-68 carries the inflammatory peptide signature which we previously investigated utilizing RSV, MHV-68 does target B-cells for infection. We are investigating the leukemogenic potential of *in utero* exposure to W followed by post-natal exposure to MHV-68 in C57BL/6 mouse pups kept immunologically naïve.

There are 3 Specific Aims and 4 associated Tasks that were outlined in the application. We have not been able to complete all of the Aims in the 1 year of the originally described work. This has been due to several issues. First, the original PI, Dr. Mark Witten, left the University of Arizona. At this departure, a change of PI was requested to Dr. Robert Clark Lantz. This change results in delays related first to the approval of the change in PI and secondarily to the subsequent move of the research related equipment and personnel from Dr. Witten to Dr. Lantz's laboratory. In addition, the main technical support person overseeing these experiments was involved in an accident that required her to be incapacitated for over a month. Because of these delays, the work could not be completed in the one year time frame. Subsequently, we requested and were granted a 1 year no-cost extension so that we could continue to perform the research as originally proposed. The results below are the results for work completed between January 1 and December 31, 2010.

Specific Aim 1 (Tasks 3 and 4). We felt that this Aim was the most important since it is designed to test the effects of virus and toxicant exposures using an in vivo animal model. So it has been given priority. It is testing the leukemogenic potential of combined exposure to JP-8 or tungsten with MHV-68 infection. Pregnant mice have been exposed to virus with subsequent exposure to JP-8 or W. Pups have been followed up to six months after birth. Each month, blood is drawn and analyzed for alterations that include anemia and neutrophilia. After 6 months animals have been sacrificed and spleens have been excised to determine splenomegaly. While this investigation is still underway, we have not noted a greater number of mice demonstrating abnormal profiles in the JP8+MHV68 group as compared to MHV68 alone. None of the mice have become anemic or morbid. The tissues from these mice in all breeding groups will be harvested by the end of February. In addition, we have noted some lymphocytosis in both the MHV-68 and W/MHV68 groups with additional alterations in blood profiles for this latter group. Additionally, a third of the W/MHV-68 group has developed upper respiratory infections while none of the other groups have developed these symptoms. However, the infection has not progressed to pneumonia or morbidity. In addition, while there are changes in the blood profiles, there are no differences between the virus alone and the virus + tungsten. We are still completing these studies as more animals are continuing to be evaluated and included in the study. However, it does not appear the combined exposures to MHV-68 and JP-8 or tungsten has any effect of parameters that would indicate alterations in the animals indicative of the development of leukemia.

Specific Aim 2 (Task 1 and 2). This Aim is designed to evaluate the effects of JP-8 and tungsten on cells that are already infected with MHV-68 (S11E cell line). We have acquired the cells and used them to develop the plaque assays that are used to determine the viral titer. All reagents are on hand and we will be performing these experiments in the next couple of months. Results will indicate if JP-8 and/or tungsten exposures can lead to viral reactivation using this in vivo system.

Specific Aim 3 (Task 3 and 4). This aim was designed to determine alterations in cellular proliferation should changes in blood cell profiles be observed in Aim 1. Since there is no indication of changes in blood cells in our experimental groups, we have not performed FACS analysis. We will perform quantitative assessment of virus only if we see splenomegaly upon sacrificing the animals.

By far the most dramatic results to date were obtained for W/RSV which included neutrophilia, splenomegaly, and morbidity conducted in a model of Greeve's Hypothesis (See Appendix). These W/RSV studies should be repeated in mice carrying t(12;21), the chromosomal translocation present in 25% of pre-B ALL and as such, is characteristic of preleukemic clones. By comparison, infection with MHV-68 and tungsten or JP-8 exposures has not resulted in similar alterations.

KEY RESEARCH ACCOMPLISHMENTS

- RSV infection and tungsten exposure will produce an inflammatory response resulting in hematological/immunological disease in pups exposed *in utero* to tungsten.
- MHV-68 infection along with exposure to JP-8 or tungsten in utero does not produce the same effects as RSV.
- There are no synergistic effects between JP-8 or tungsten and MHV-68 on virus reactivation

REPORTABLE OUTCOMES

Fastje, C.D., Harper, K., Sheppard, P.R., Witten, M.L. In Utero Exposure to Sodium Tungstate and Post-Natal Exposure to Respiratory Syncytial Virus Results in Hematological/Immunological Disease in Mice: Implications for Childhood Leukemia. Chemico-Biological Interactions. In Review.

Abstract and poster from Experimental Biology Annual meeting, April, 2010 (see Appendix).

CONCLUSION

From the data collected to date, neither exposure to JP-8 nor tungsten cause reactivation of MHV-68 virus in an animal model. Under similar exposure conditions, respiratory syncytial virus did lead to altered blood profiles and alterations in the spleen. This indicates that there may be differences in the responses based on the types virus and/or the cell types that they

infect. The overall response therefore, may be specific for particular viral interactions and is not just a general effect of the JP-8 or tungsten on inflammatory responses.

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APPENDICES

Submitted manuscript under review at Chemico-Biological Interactions

Experimental Biology 2010 Abstract

In Utero Exposure to Sodium Tungstate and Postnatal Exposure to Respiratory Syncytial Virus Results in Hematological/Immunological Disease in Mice: Implications for Childhood Leukemia

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Abstract

Little of the etiology of childhood leukemia is known. Strong evidence indicates that childhood leukemia is a genetic disease originating *in utero.* Because risk factors are hypothesized to include environmental exposure, genetic predisposition, and viral infection with a probable multi-factorial etiology, we profiled and compared the environmental exposures in two concurrent, childhood leukemia clusters and modeled the exposures shared in common by the leukemia clusters in C57BL/6 mice utilizing prenatal exposures. This previous investigation has suggested *in utero* exposure to sodium tungstate (W) may result in hematological/immunological disease via genes associated with viral defense. **Hypothesis:** We hypothesize that prenatal exposure to an environmental chemical, held in common by communities experiencing elevated rates of leukemia, but not experienced by geographically similar control communities, results in genetic changes associated with increased susceptibility towards virus-induced leukemogenesis. Our working hypothesis for this study is (1) in addition to spontaneously and/or chemically generated chimeral genes forming pre-leukemic clones, *in utero* exposure to W increases genetic susceptibility to viral influence(s); (2) postnatal exposure to a virus possessing the ^{1}FXXKXFXXA/V^{9} peptide motif will cause an unnatural immune response encouraging proliferation in the B-cell precursor compartment. **Results:** Inoculation of C57BL/6 mice with Respiratory Syncytial Virus (RSV) within two weeks of weaning was associated with a neutrophil shift in 56% of 5-month old mice. When the RSV inoculation was combined with an *in utero* exposure to W (W/RSV), true neutrophilia resulted and was accompanied by progressive anemia, splenomegaly (p=0.0406, 0.0184, 0.0108 for C, W and RSV, respectively) and death in a subset of the mice. **Conclusion:** *In utero* exposure to sodium tungstate and post-natal exposure

to RSV in mice results in hematological/immunological disease. Further research to characterize this potential leukemia mouse model is strongly warranted.

Key Words: tungsten; Respiratory Syncytial Virus; childhood leukemia

Abbreviations:

W – tungsten, contextually represents sodium tungstate ($Na_2WO_4 \cdot 2H_2O$)

RSV – Respiratory Syncytial Virus

1. Introduction

The toxicology of tungsten in physiological systems is under scrutiny because of the co-occurrence of temporal elevations in tungsten concentrations with the elevation in rates of childhood leukemia in Fallon, NV [1-4]. Historically, tungsten compounds have been reported to be toxicologically and biologically inert including in *Escherichia coli*, except for effects on clpB and osmY stress promoter-genes and inhibition of enzymes with nucleic-acid substrates [5]. Recent research has demonstrated variable degrees of *in vitro* toxicological effects in phosphate-dependent pathways associated with cell-cell communications for exposure to sodium tungstate (W) and/or its polymerized form(s) created down-stream from the point of exposure [6].

Little of the etiology of childhood leukemia is known; evidence indicates that childhood leukemia is a genetic disease originating *in utero* (e.g. [7-9]). Leukemogenic risk factors are hypothesized to include environmental exposure, genetic predisposition (primarily the prenatal formation of chimeral genes resulting from chromosomal translocations), and viral infection with a probable multi-factorial etiology (reviewed in [10, 11]). We are profiling and comparing the environmental exposures in several childhood leukemia clusters [12] in the Southwestern United States primarily at Fallon, Nevada [10, 13-15], but also in Sierra Vista, Arizona [13, 16]. Utilizing prenatal exposures, we are modeling in C57BL/6 mice those exposures shared in common by the leukemia clusters. We hypothesize that prenatal exposure to an environmental chemical, held in common by communities experiencing elevated rates of leukemia, but not experienced by geographically similar control communities, results in genetic changes associated with increased susceptibility towards virus-induced leukemogenesis.

A summary of our environmental profiling of Fallon, Nevada indicates that the unique portion of the atmospheric, metallic particulate matter is significantly elevated concentrations of

cobalt and tungsten which co-vary as a function of time [17, 18]. The temporal time study, utilizing dendrochemistry of tree rings, indicates that not only are tungsten concentrations elevated as compared to control communities ($p=0.06$ for the 2002 tree-ring group), but that tungsten concentrations were about the same as the control communities a decade prior to the onset of the childhood leukemia cluster ($p>0.50$ at the 1990 tree-ring group) [18].

Previously, we exposed C57BL/6 mice to sodium tungstate while *in utero* and examined the resultant transcriptome for differential changes in genes known to be consistently altered in acute lymphoblastic leukemia [19] and for significantly altered pathways and networks that hypothetically, may contribute to leukemogenesis. Results suggest that *in utero* exposure to sodium tungstate may result in hematological/immunological disease via genes associated with viral defense (Table 1 and [17]).

2. Theory

A consensus of the developing hypotheses regarding childhood leukemogenesis involves a two-step etiology that is focused on genetic mutations and their resulting molecular effects:

- prenatal mutations involving translocation of genes encoding transcription factors and/or rearrangements in T-cell receptor (TCR) genes or Immunoglobulin Heavy Chain (IgH) genes are generated (e.g. [7-9]);
- post-natal mutations endowing the ability to halt differentiation [if not already endowed by previous mutation(s)] and apoptosis are acquired (e.g. [20, 21]).

We have utilized this pre-natal/post-natal sequence as an exposure protocol to test leukemogenic hypotheses developed from the results of the gene microarray study of *in utero* exposure to sodium tungstate. The hypothesis presented here is -

- In addition to spontaneously and/or chemically generated chimeral genes forming pre-leukemic clones, *in utero* exposure to tungsten-compounds increases genetic susceptibility to viral influence(s);
- Postnatal exposure to a virus possessing the ^{1}FXXKXFXXA/V^{9} peptide motif (where X is unknown, F is phenylalanine, K is lysine and A/V is either alanine or valine in the 9th position), will cause an unnatural immune response encouraging proliferation in the B-cell precursor compartment [22].

3. Methods and Materials

3.1 Mice

Eight week-old, C57BL/6 male and female mice were purchased through an IACUC approved protocol. After acclimating to the new environment, a transponder (Bio Medic Data Systems; DAS-6006; IMI-1000) was injected at the nape of the neck of each female mouse and each mouse randomly assigned to an exposure group. The mice were housed, cared for and bred in sterile, microisolation in a Biolevel 2 facility by University of Arizona Animal Care.

3.2 Experimental Design

Because the etiology of leukemia is not known, we provided a blanket exposure to W utilizing Fallon, NV as the model source. Female mice were assigned to an exposure group (N=6/group and replicated a second time for a total of 12/group), received a pre-gestational exposure to sodium tungstate ($Na_2WO_4{\cdot}2H_2O$, Acros Organics, 99+%, ACS, Lot A0260722, IN, USA) to decrease the expression of the SUOX gene prior to conception [14, 23] bred and

continued gestational exposure to sodium tungstate (W) through aerosol and water at concentrations to model the environmental exposures a woman residing in Fallon, NV would inhale and consume during 3 months of attempting to become pregnant and 40 weeks of gestation and then normalized to a mouse (0.3% of the human value with obligate nasal-respiration and the associated enhanced mucosal-elevator taken into consideration). Following weaning, the progeny were lightly anesthetized and exposed to Respiratory Syncytial Virus (RSV) between 28 – 35 days of age during the first study and 21-28 days of age during the second study. Although human fathers residing in Fallon also breathe the air and drink the water, mouse sires were not exposed to tungsten because a previous study indicated that exposure to sodium tungstate inhibited spermatogenesis [24]. Experimental groups consisted of longitudinal controls to monitor for spontaneous disease, tungsten-only to determine whether *in utero* exposure to W is leukemogenic later in childhood, RSV-only to ascertain the leukemogenic potential of RSV alone in an unmodulated immune system [22] and W/RSV to determine whether the changes in the transcriptome associated with *in utero* exposure to tungsten may increase susceptibility to RSV influences and promote leukemogenesis. All pups were kept immunologically unmodulated (i.e. housed in sterile microisolation).

3.3 Tungsten Exposures

The dams were exposed to sodium tungstate through water (15 ppm, *ad libetum*) and aerosol (modal concentrations: total W 197.39 mg/m^3, PM_5 1.11 mg/m^3). A 187 g/L exposure solution was made by autoclaving and dissolving the tungsten into sterile physiological saline and bringing to a more neutral pH (8.0) prior to being nebulized and vacuum-drawn through a nose-only INTOX inhalation chamber (Albuquerque, NM, USA) at 20 L/min for an average of

8.5 preconception/gestational 45-minute exposures. (Note: The solution was created two weeks prior to actual inhalation exposures and allowed to sit at room temperature. Interestingly, newly made sodium tungstate in medium had no differential effect on cells, but aged tungstate in medium demonstrated significant toxicological effects [25] suggesting the presence of polyoxotungstates [17] which are more toxic than monotungstates (reviewed in [26]).) A cascade impactor attached to the INTOX inhalation chamber sampled the portion of particulates less than 5μm (PM_5), which is the portion most likely to achieve pulmonary deposition in the mice. We were able to nebulize a total of 2.0710 g of tungsten during pre-conception/gestational exposures and assume a total pulmonary deposition of 0.003275 g PM_5.

3.4 RSV Exposure

Because the protein product of the G gene of Respiratory Syncytial Virus (RSV) possesses the FXXXFXXA/V peptide motif within two T-cell epitopes that are restricted by the HLA-DP2/DP4 [27, 28] supertype significantly associated with B-cell precursor Acute Lymphoblastic Leukemia (ALL) [22], we lightly anesthestized 21 - 35 day old mouse pups and inoculated the nasal cavity with 10μL of human RSV in medium for a total exposure of $1x10^6$ pfu [29]. No pathology was observed in the mice. The hRSV A2 strain, propagated in Hep-2 cells, was a kind gift from Dr. Kevin Herrod of the Lovelace Respiratory Research Institute in Albuquerque, NM, USA.

3.5 End Measures

Peripheral hematology was evaluated utilizing complete blood counts with a differential obtained from an automated HEMAVET 850 Multispecies Hematology Analyzer (Drew

Scientific Inc., Oxford, CT, USA). Spleen tissue was massed and splenic ratio calculated as spleen mass per body mass. Spleens and femurs were preserved in 10% formalin and submitted to Tissue Acquisition and Cellular/Molecular Analysis Shared Services (TACMASS) of the Arizona Cancer Center at the University of Arizona for embedding, slide preparation and H&E staining. Histopathological interpretation was conducted by University of Arizona Animal Pathology Services. Digital images were created with a Nikon LaboPhot-2 microscope Paxcam 3 camera and PAX-it Digital Image Management & Image Analysis at 20x magnification by TACMASS.

3.6 Statistical Analysis

Data was collected and combined from two separate investigations each producing similar results (true neutrophilia leading to morbidity/death). Because studies have indicated that an RSV infection as an infant increases the susceptibility to repeat respiratory ailments over the subsequent decade (e.g. [30, 31]), we hypothesized that exercise induced asthma may be associated with acute onset of childhood leukemia. Therefore, we originally included a hypoxic factor in these investigations. However, due to inconsistent methods of administering the challenge, the data were not included in the analysis. Statistical analysis was performed with a Mann-Whitney test utilizing Minitab version 15.1.1.0. Data are presented as medians with interquartile ranges and outliers indicated. Significance was accepted when $p \leq 0.05$.

4. Results

Longitudinal controls and tungsten mice did not exhibit pathological indicators. RSV inoculation within two weeks of weaning was associated with a neutrophil shift in 56% of 5-

month old mice. When the RSV inoculation was combined with an *in utero* exposure to W (W/RSV), true neutrophilia resulted and was accompanied by progressive anemia, splenomegaly and death in a subset of the mice (Table 2).

4.1 Peripheral Hematology

W, RSV and W/RSV demonstrated significantly elevated neutrophil counts as compared to the longitudinal controls ($p=0.0162$, 0.0081, 0.0059 for RSV, W and W/RSV, respectively). However, the first and third quartiles for all experimental groups were within the normal range (0.1 – 2.4 k/µl) except for RSV-only whose third quartile was 2.76 k/µl. Additionally, W/RSV possessed an extreme outlier (Table 2). No significant differences were demonstrated between RSV, W and W/RSV.

The percentage of neutrophils contributing to the total WBC count was significantly greater for all three exposure groups as compared to the controls. However, the first and third quartiles for all experimental groups were within the normal range (6.6 % - 38.9 %) except for RSV-only whose third quartile was 44.67%. Additionally, W/RSV possessed an extreme outlier for whom 76.3% of the WBC was neutrophils. No significant differences were demonstrated between W, RSV and W/RSV (Figure 1A).

While the percentage of neutrophils increased significantly, there was no associated increase in WBC counts for mice exposed to RSV-only. No group demonstrated a significant increase in WBC counts as compared to the controls or to each other. First and third quartiles for all experimental groups were within the normal range (1.8 – 10.7 k/µl) except for W/RSV whose third quartile was 12.4 k/µl (Figure 1B). A greater n-value would be needed to obtain significance. Additionally, a confounding variable of age at RSV inoculation is thought to be

influencing the data. Pups inoculated at 21 days as opposed to 35 days of age demonstrated greater pathology. For the subgroup of W/RSV mice exhibiting pathology, leukocytosis began at 2-3 months of age and continued until death/morbidity at 5-6 months of age.

4.2 Splenomegaly

Splenic ratios for mice exposed to W or RSV did not vary significantly from the controls or from each other. Splenic ratios for W/RSV mice were significantly larger as compared to all other groups ($p=0.0406$, 0.0184, 0.0108 for C, W and RSV, respectively) (Figure 2A). The colors/hues of the spleens were not consistent in mice demonstrating splenomegaly and were not the same color as the control spleens (e.g. Figure 2B).

4.3 Histology

Histologic slide preparation and interpretation was conducted with both spleen and bone marrow tissues from the two W/RSV mice exhibiting the greatest degree of splenomegaly and compared with a longitudinal control mouse. The control exhibited a 1:1 ratio of erythropoietic and granulopoietic precursor cells with all stages represented. Both W/RSV mice demonstrated consistent histology, a 1:20 ratio of erythropoietic to granulocytic precursors with all stages represented (Figure 3). Additionally, marked thrombopoiesis was reported in both spleen and bone marrow tissues.

5. Discussion

We hypothesize that prenatal exposure to an environmental chemical (W), held in common by communities experiencing elevated rates of leukemia, but not experienced by

geographically similar control communities, results in genetic changes associated with increased susceptibility towards virus-induced leukemogenesis. Previous results have suggested that *in utero* exposure to sodium tungstate may result in hematological/immunological disease via genes associated with viral defense [17]. This study provides additional evidence that post-natal exposure to RSV can promote shift neutrophilia ($p=0.0015$ as compared to controls, but not significantly different from W or W/RSV) at a later time with most hematological measures remaining within normal parameters. When this RSV-induced T-cell activation is combined with *in utero* exposure to W, leukocytosis (not significant) and splenomegaly results ($p=0.0406$, 0.0184, 0.0108 for C, W and RSV, respectively) culminating in morbidity/death in a subset of the mice.

5.1 The Leukemogenic Hypothesis

A general consensus of oncogenesis is that cancer results from sequential, genetic mutations commonly referred to as hits. Strong evidence supports that childhood leukemia results from at least one of these hits occurring *in utero* (e.g. [7-9]). This first hit produces preleukemic clones.

Cytogenetic/molecular studies characterizing these mutations and other markers in leukemia cells, epidemiological investigations characterizing leukemia patients and geobiological studies characterizing leukemia communities have contributed to the development of several leukemogenic hypotheses supporting a viral component, whether directly or as a response to an infectious agent. Kinlen's hypothesis [32, 33] focuses on population mixing; Greaves' hypothesis [34, 35] suggests that a common infection promotes the second hit and/or proliferation, but primarily postulates that a delay in the normal exposure of the immune system

to infection delays identification of cells carrying the primary hit thereby increasing the total number of preleukemic cells available for the second hit; Smith's hypothesis [36] suggests that an *in utero* exposure to an infection is associated with the later development of leukemia. Utilizing technological advances in medical geography, we have identified exposure to tungsten as unique to leukemia clusters and *in utero* exposure as potentially capable of interfering with normal immune response to viruses in neonatal mice. We incorporated aspects of the published postulates into several working hypotheses, the first of which we have presented here and which focuses on Greaves' hypothesis. We limited the infectious agents to viruses, but the evidence for direct viral involvement in leukemogenesis is weak (reviewed in [37, 38]). Therefore, we incorporated the work being conducted in delayed immune response to an infectious agent (Greaves' Hypothesis) which has identified the peptide motif, ^{1}FXXKXFXXA/V^{9}, as the antigen that left its signal on the HLA-DP2/DP4 loci significantly associated with B-cell precursor ALL [22]. Additionally, W exposure significantly altered antigen processing and presentation pathways, suggesting a role in altering immune response to an antigen [17]. Finally, Taylor (2008) speculated that RSV may be the antigen significantly associated B-cell precursor ALL because the G protein of RSV contains two T-cell epitopes that are restricted by HLA-DP2/DP4, elicits CD4+T-cell responses [27, 28], and RSV is highly contagious, but weakly pathogenic.

Based upon Greaves' hypothesis, Taylor's speculation, and our W data, we developed the following working leukemogenic hypothesis: Preleukemic clones are produced spontaneously during *in utero* growth and possibly promoted by *in utero* exposure to W. Additionally, *in utero* exposure to W promotes susceptibility to viral influence(s). Exposure to RSV initiates an immune response activating T-cells. W influences dysregulates this T-cell

response unleashing a cytokine cascade [17] that inhibits normal hematopoiesis resulting in immune suppression [38]. The preleukemic clones are immersed in a microenvironment of inflammatory cells, growth factors and agents producing oxidative stress all of which both promotes proliferation and enhances the probability of a secondary hit [38]. One clone obtains a selective advantage and begins to proliferate into overt leukemia. Recently, dysregulated T-cells in mice, as part of an inflammatory response, resulted in lymphocytic leukemia [39].

5.2 *Neutrophilia*

Two different types of neutrophilia were observed in the mice. RSV-only and W-only mice demonstrated significantly elevated neutrophil counts ($p=0.0015$, $p=0.0265$ for RSV and W, respectively) compared to control mice without an associated increase in total leukocytes (shift neutrophilia). W/RSV mice demonstrated significant neutrophilia compared to controls ($p=0.0015$), which included a corresponding increase in total leukocyte counts (true neutrophilia). Because all exposures were completed by 5 weeks of age and the blood draws were performed on all mice, the W-only and RSV-only groups of mice at 5 months experienced the same level of stress as controls and therefore, the significant difference in nuetrophil counts is probably not associated with chronic stress, but attributable to the exposures. RSV has long been known to activate neutrophils (e.g.[40]), however, the literature does not report such an effect for W exposure.

W-metal has been reported to decrease inflammation, but this was specific to xanthine oxidase activity associated with TNF-α and did not influence the other mediators of inflammation tested in brain endothelial cells [41]. More recent reports of *in vitro* W exposure in human peripheral blood lymphocytes and acute leukemic monocytes indicated reduced cytokine

production and altered cell cycle progression [42]. Ingestion of W for 28 days in mice, followed by an immune challenge, dose-dependently reduced activation/proliferation of cytotoxic T-cells, helper T-cells and positive NK cells in spleen tissue, but not blood [43]. Myeloid cells were not affected. These studies support the hypothesis that exposure to W influences immune system responses to a challenge. Additionally, *in utero* exposure may alter the nature of this immunological effect.

Why did inoculation with RSV at 1 month still produce shift neutrophilia at 5 months of age? RSV infections of the lower respiratory tract in infants are associated with recurrent wheezing, the incidence of which slowly declines over the subsequent decade [30, 31], suggesting latency and periodic reactivation of the virus. Infective RSV can be recovered from T-cell depleted murine lungs up to 150 days post-infection, however RSV's persistence was not associated with evasion by mutation [44]. The effect of a persistent presence, whether of an actual hidden population of virus or retention of viral antigen, has been hypothesized to influence the immunological functions/patterns in the still developing immune system of the infant (reviewed in [45]), such as altering chemokine and cytokine production [46], with the end result of the virus and/or the immune system promoting chronic inflammation [47]. The timing of the post-natal RSV inoculation in this study (21 days old vs. 35 days old) may have influenced some characteristics of this inflammatory response as has been demonstrated for neurogenic inflammation in lungs for 2 week vs. 12 week old rats in association with RSV inoculation [48].

5.3 Diagnosis

Splenomegaly can result from infections, hemolytic anemia, autoimmune disease, or various neoplasias [49]. Neutrophilia can result from inflammation, myloid leukemias or either

viral or bacterial infections. Either a viral infection or a chronic, severe bacterial infection can produce lesions similar to those observed in the bone marrow and spleen tissue samples. The two mice from which the tissue images were obtained demonstrated progressive anemia, but not thrombocytopenia, suggesting they may have been immunocompromised, and they both possessed bacterial abscesses. Two additional female mice were cage mates and had normal hematology reports with spleen and body masses similar to the controls, further suggesting that the two mice presenting with anemia/splenomegaly were immunocompromised providing an opportunistic environment. The combination of immunosuppression, neutrophilia and splenomegaly indicates neoplasia must be considered [50]. Further research is needed to identify and characterize the W/RSV-associated hematological/immunological disease(s). A myeloproliferative disorder (MPD) was the initial presentation in mouse hematopoietic cells with deletion of the Pten (phosphatase and tensin homolog) gene which was followed by acute T-lymphoblastic leukemia in 76% of the mice and AML in 26% [51]. The MPD was described as beginning as early as 1 month old with a myeloid shift of increased neutrophil counts which chronically increased until true neutrophilia resulted with the associated increase in leukocyte counts and culminated with leukemic blast invasion into hematopoietic and non-hematopoietic organs [51].

6. Conclusion

This study provides evidence indicating *in utero* exposure to sodium tungstate (and/or its polymerized forms) and a post-natal exposure to RSV produces a possible leukemia mouse model. However, further research is needed to characterize the model.

Conflict of Interest

Sheppard and Witten have provided documents, data, and testimony in case CV03-03482, Richard Jernee et al. vs Kinder Morgan Energy et al., Second Judicial District Court of Nevada, Washoe County, which is related to the childhood leukemia cluster of Fallon, NV.

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Captions for Figures

Figure 1. Percent Neutrophils (A) and WBC counts (B) for 5-month old C57BL/6 mice exposed to W while *in utero* and/or to the Respiratory Syncytial Virus within two weeks of weaning. First and third quartiles with a median bar, whiskers and extreme outliers (●) are indicated. *C indicates a significant difference from the longitudinal controls (p=0.0015, 0.0265, 0.0015 for RSV, W and W/RSV, respectively).

Figure 2 A. Splenic ratios for 5-month old C57BL/6 mice exposed to W while *in utero* and/or to the Respiratory Syncytial Virus within two weeks of weaning. First and third quartiles with a median bar, whiskers and extreme outliers (●) are indicated. *C indicates a significant difference from the longitudinal controls, *W from tungsten, *RSV from Respiratory Syncytial Virus. Figure 2 B. Image of control spleen as compared to W/RSV spleen harvested from mouse for which we did not obtain a blood sample (Table 2).

Figure 3. Spleen and bone marrow images obtained from H&E slides with x20 magnification (A) control femur with bone marrow (B) W/RSV femur with bone marrow (C) control spleen (D) W/RSV spleen.

Table 1. Description of Genes Associated with Hematological/Immunological Disease and Cell Death in a Network Generated from Genes Differentially Expressed > 5-fold in Spleen Tissue.

(Study reported in [17] and gene descriptions obtained from [52, 53].)

Molecules[a]	Name; Known Function(s)
ABCB1B, ACAA1,	acetyl-Coenzyme A acyltransferase 1; beta oxidation system of the peroxisomes
ANGPTL4,	angiopoietin-like 4; inhibition of vascular activity preventing metastasis
ANKRD25,	KN motif and ankyrin repeat domains 2; matrix remodeling
ATG7, Cbp/p300,	autophagy related 7 homolog; fusion of peroxisomal and vacuolar membranes
CDKN2A, cyclic AMP, DCTN4,	dynactin 4; linking dynein and dynactin to the cortical cytoskeleton
dehydroepiandrosterone sulfate, ↓DUSP2,	dual specificity phosphatase 2; Regulates mitogenic signal transduction by dephosphorylating both Thr and Tyr residues on MAP kinases ERK1 and ERK2
↓DUT (includes EG:1854),	deoxyuridine triphosphatase; nucleotide metabolism - produces dUMP, the immediate precursor of thymidine nucleotides; decreases intracellular concentration of dUTP preventing incorporation of uracil into DNA
↓GABARAP,	GABA(A) receptor-associated protein; interaction with the cytoskeleton
GH1, HMGA2,	high mobility group AT-hook 2; transcriptional regulator, cell cycle regulation
HMGC52,	Not found

hydrogen peroxide, ↓IRF3,	interferon regulatory factor 3; Functions as a molecular switch for antiviral activity
LGP2,	DEXH (Asp-Glu-X-His) box polypeptide 58**;** Participates in innate immune defense against viruses
↓LIPE, MCHR1,	lipase, hormone-sensitive**;** primarily hydrolyzes stored triglycerides to free fatty acids; steroid hormone production
PHF20,	PHD finger protein 20**;** possible transcription factor
↓PPAP2A,	phosphatidic acid phosphatase type 2A; dephosphorylating lysophosphatidic acid (LPA) in platelets which terminates signaling actions of LPA.
PPARG, ↓RAD23A,	RAD23 homolog A; post-replication repair of UV-damaged DNA
↓RPL21,	ribosomal protein L21; ribosomal protein that is a component of the 60S subunit
SLC31A2,	solute carrier family 31 (copper transporters), member 2**;** low-affinity copper uptake
SLC7A11,	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11; anionic form of cystine is transported in exchange for glutamate
SLCO1, SLCO1B3,	solute carrier organic anion transporter family, member 1B3**;** Mediates the Na(+)-independent transport of organic anions such as methotrexate
SOD2,	superoxide dismutase 2, mitochondrial; destroys toxic radicals normally produced within the cells

↑TP53INP1,	tumor protein p53 inducible nuclear protein1; promotes p53/TP53 phosphorylation on 'Ser-46' and subsequent apoptosis in response to double-strand DNA breaks
↓UQCRH,	ubiquinol-cytochrome c reductase hinge protein; component of the ubiquinol-cytochrome c reductase complex that is part of the mitochondrial respiratory chain
↓WDR5,	WD repeat domain 5; contributes to histone modification; as part of the MLL1/MLL complex it, is involved in methylation and dimethylation at 'Lys-4' of histone H3
ZMYND19	zinc finger, MYND-type containing 19**;** binds to the C terminus of melanin-concentrating hormone receptor-1

[a]Genes colored red were up-regulated and genes colored green were down-regulated in the gene microarray analysis. Genes in black capitals were differentially expressed less than 5-fold.

Table 2. 5-Month Old Mice Demonstrating Pathology Following Post-natal Exposure to RSV-only (RSV) and *In Utero* Exposure to W (W/RSV).

Group	Gender	WBC(k/µl)	Neutrophils(k/µl)	Lymphocytes (%)	Other Hematopathology	Spleen (g)/Body (g)
RSV	Female	9.08	4.05	42.83	None	0.1022/21.1
RSV	Male	8.06	4.15	43.03	None	0.0835/22.7
RSV	Male	6.5	2.46	57.95	None	0.0914/25.0
RSV	Female	8.6	2.92	57.64	None	0.0818/19.5
RSV	Female	4.68	2.6	38.88	None	0.0826/19.2
W/RSV	Female	12.4	2.54	73.84	leukocytosis	0.1282/21.5
W/RSV	Female	20.68	15.79	15.55	Anemia, leukocytosis	0.2384/17.1
W/RSV	Female	*	*	*	*	0.252/17.2
W/RSV	Female	4.22	1.42	55.80	monocyte shift-only	0.1771/23.6

*mouse expired prior to blood draw

Figure 1A & 1B without caption

Figure 1A

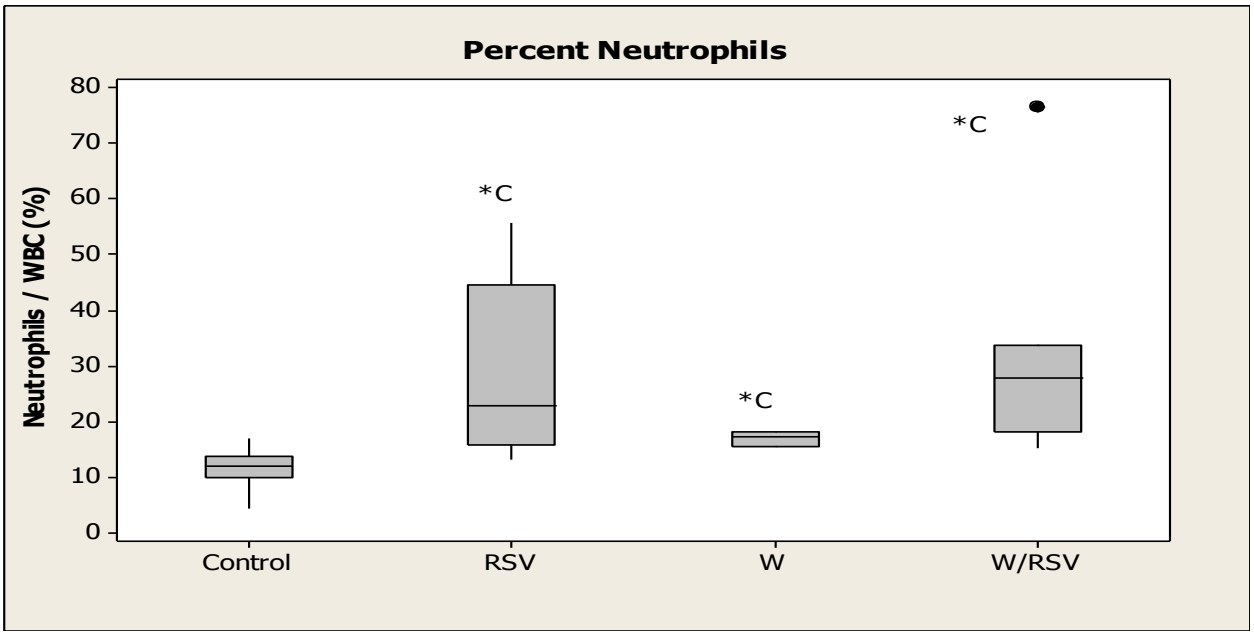
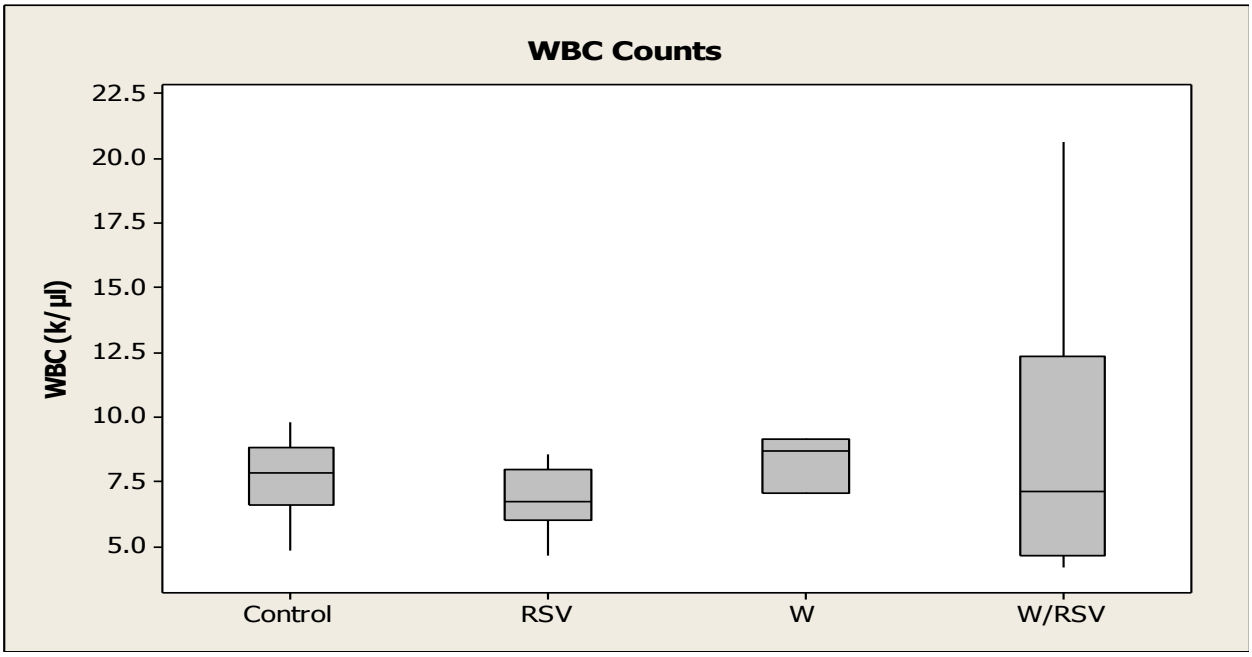


Figure 1B



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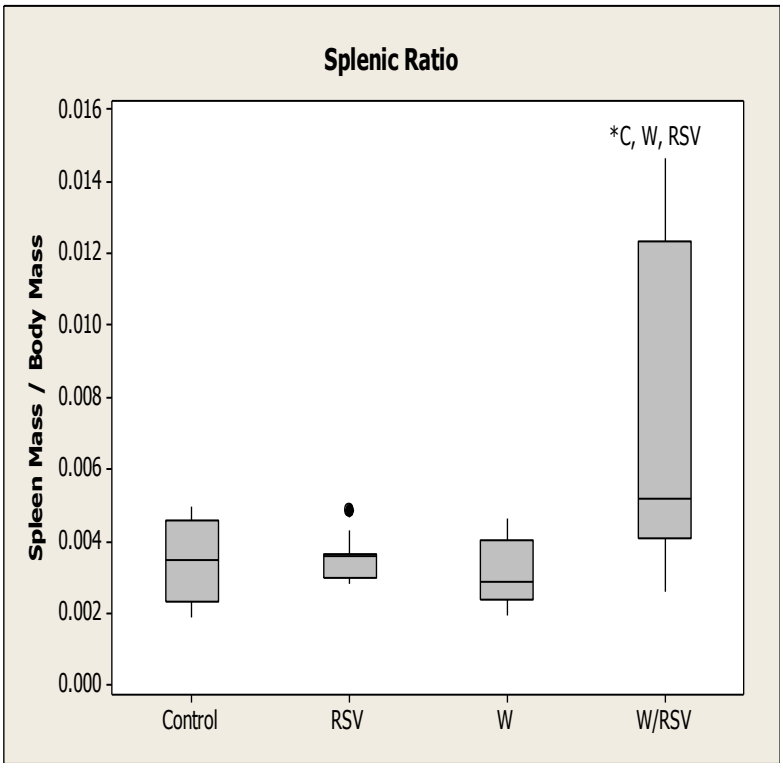

Figure 2B without caption
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Figure 3A without caption
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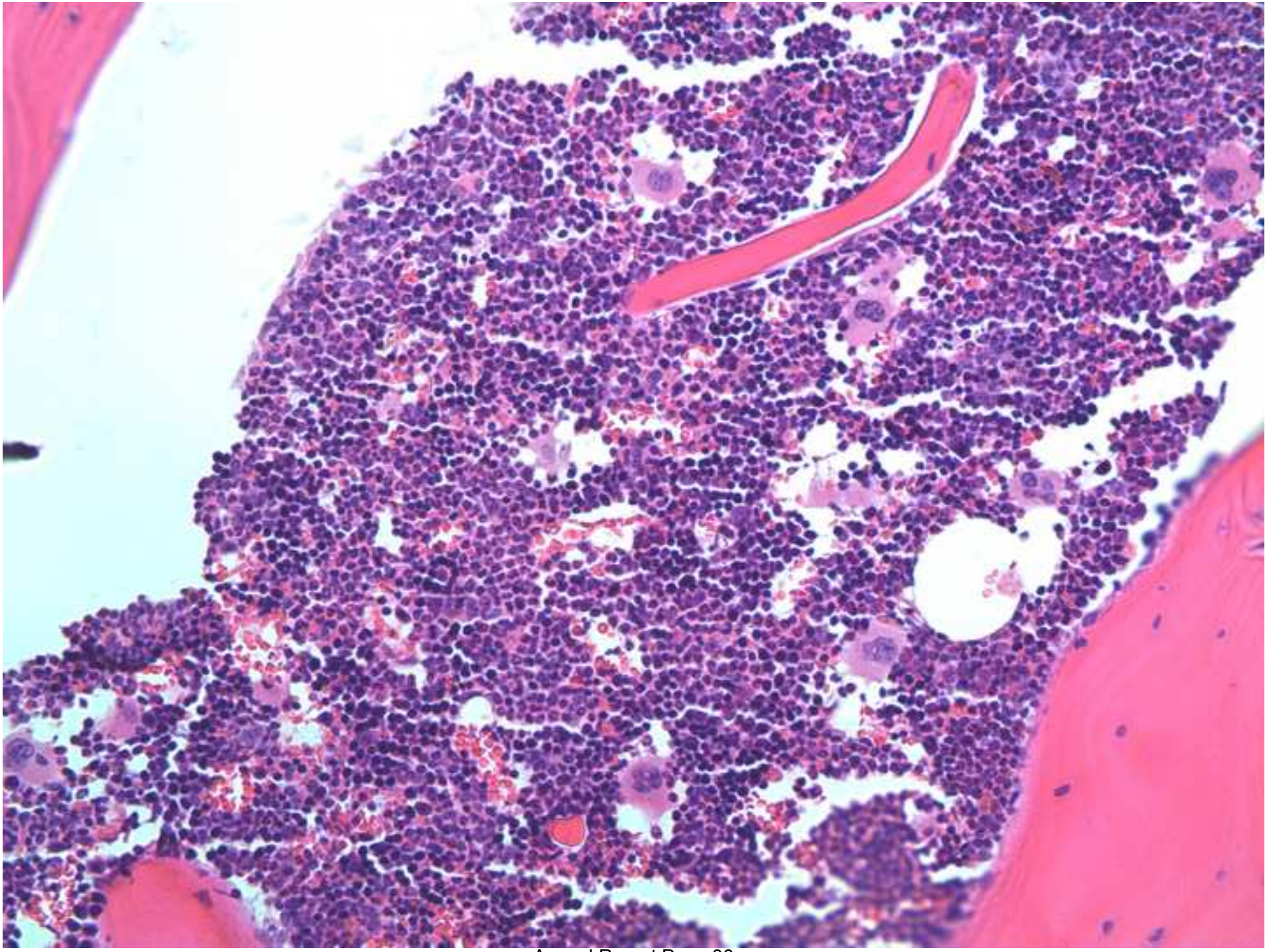

Figure 3B without caption
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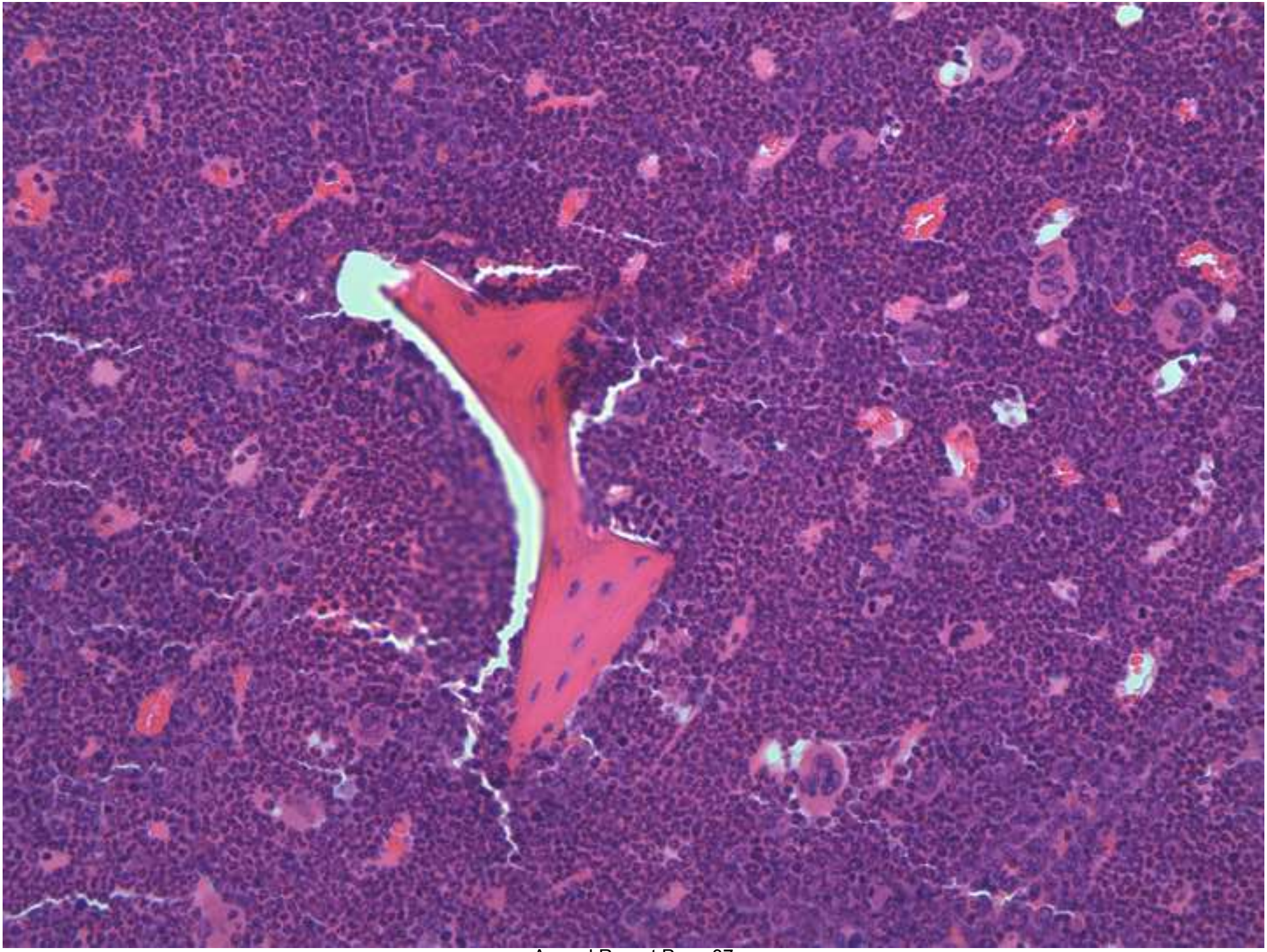

Figure 3C without caption
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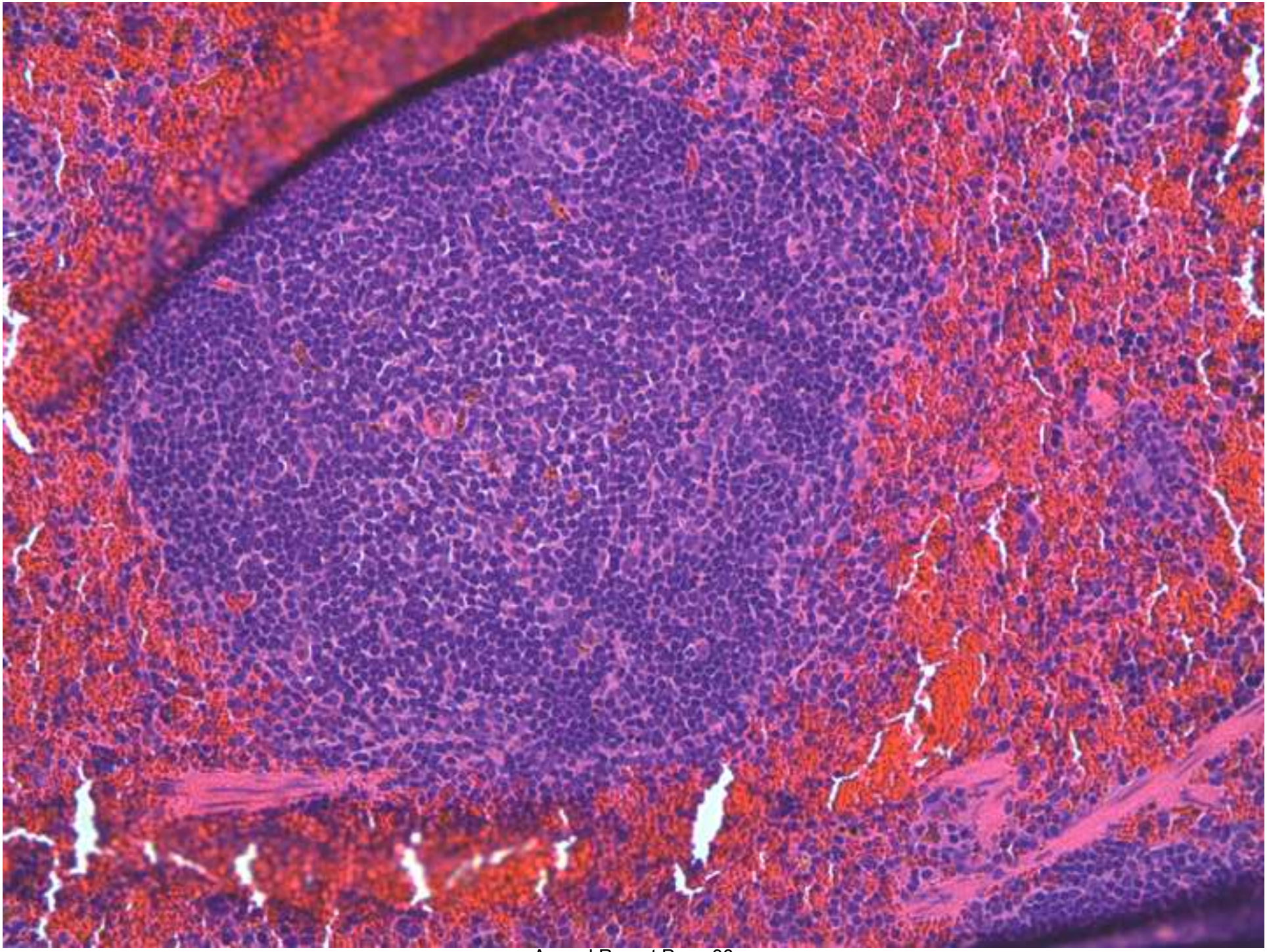

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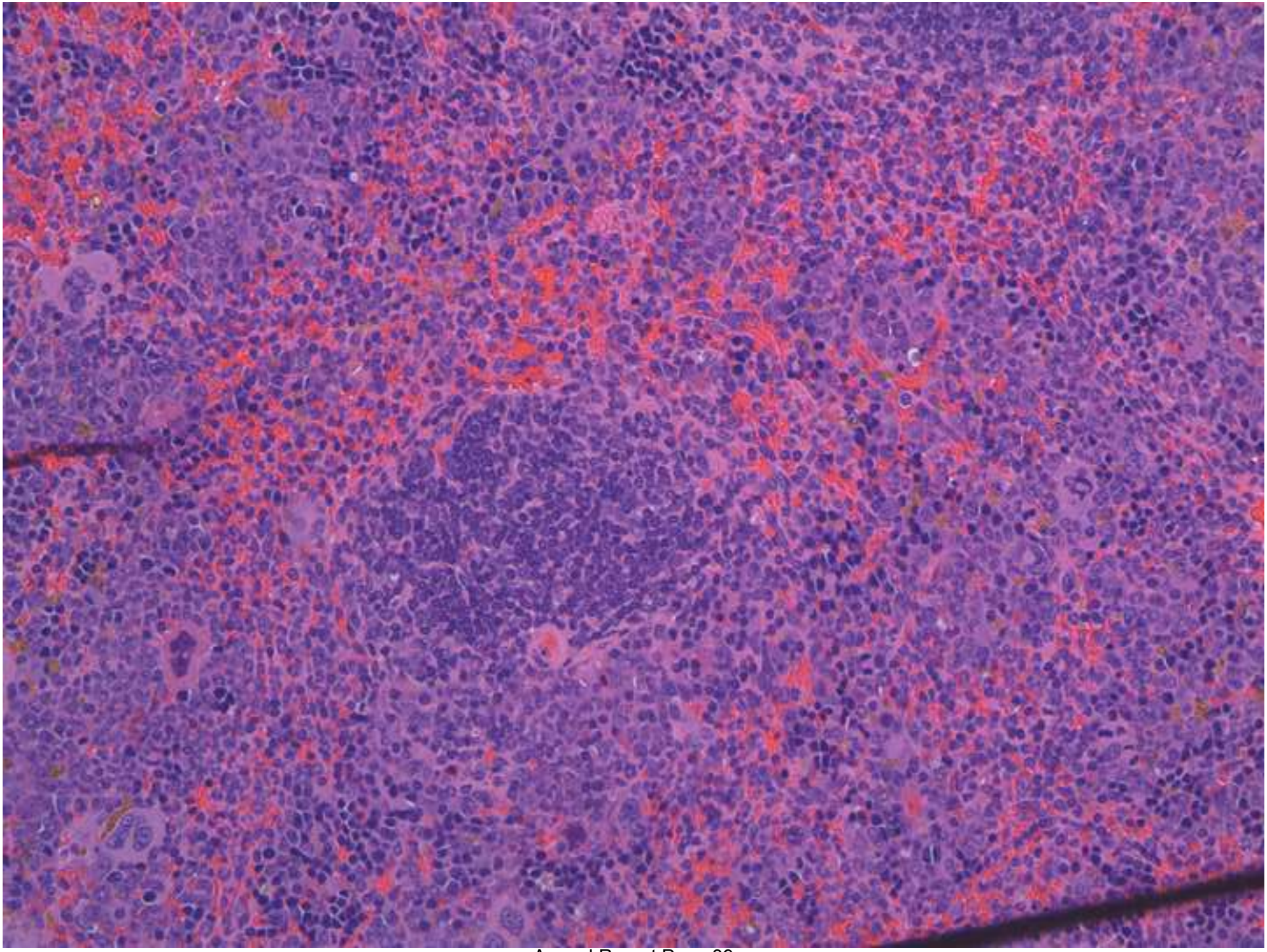

Experimental Biology 2010

Program No. 563.13

Investigating a 3-Step Leukemogenic Hypothesis in C57BL/6 Mice: Tungsten, Respiratory Syncytial Virus and Hypoxia

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Abstract

Significantly elevated concentrations of tungsten have been demonstrated in the atmospheric particulate matter in multiple communities experiencing elevated rates of childhood leukemia, but not in their respective control communities. Prenatal exposure to sodium tungstate in C57BL/6 mice via aerosol and drinking water significantly decreased the transcriptome expression of Dmbt1, a protein which functions to aggregate bacteria and viruses in lung mucosa and saliva. Additionally, analysis of gene microarray data produced a significant network associated with hematological/immunological disease focused on genes involved in viral defense. We hypothesize that the etiology of environmentally-induced leukemia is in utero exposure to metals such as tunmgsten (W) which may increase susceptibility to viral influences, post-natal exposure to Respiratory Syncytial Virus (RSV), and triggered by a hypoxic event. End measures include qRT-PCR of RSV genes F & G during the fastigium of the infection, RT2-PCR for genes associated with immunological function, and a complete blood count with a differential. Mice in the W/RSV/Hypoxia group developed neutrophilia post-hypoxic challenge and at five minths developed lymphocytosis contributing to leukocytosis.